

## Multifunctional Nanorods for Gene Delivery

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The possibility of achieving control of size and composition by inorganic synthesis recently prompted us to evaluate the potential of novel multi-segment metallic carriers in gene delivery.[1] Deposition of the Au/Ni nanorods was achieved by template synthesis. This technique involves electrochemical deposition into a non-conducting membrane with an array of cylindrical pores and has been used for the synthesis of a wide range of materials and structures.[2, 3] Subsequent etching of the membrane results in metallic nanorods of defined dimensions. This method is advantageous because it can be changed with ease for the deposition of different metallic segments.

The nanorods are 100 nm in diameter and 200 nm in length with a 100 nm gold component and a 100 nm nickel component. Using linkages with end groups that selectively bind to gold or nickel, plasmid-DNA and transferrin, a cell receptor protein is attached to the different components. The transferrin immobilization is achieved by converting a small proportion of the primary amine groups of transferrin to sulfhydryl groups, which is known to have a strong affinity for gold.[4] DNA is attached to the nickel segments by suspending the dual component nanorods in a 0.1 M solution of 3-[2-aminoethyl] dithio] propionic acid (AEDP). The carboxylic acid end-group of AEDP binds to the nickel component of the nanorod because of its strong affinity to nickel oxide.[5] This results in the surface presentation of primary amine groups spaced by a reducible disulfide linkage. Plasmid encoding luciferase or plasmids encoding the green fluorescent protein (GFP) is conjugated to the AEDP bound to the nickel components of the nanorods at pH 5.7 by electrostatic interactions. The surface plasmid concentration, determined from UV-Visible absorbance spectroscopy, is approximately  $4 \times 10^{12}$  molecules  $\text{cm}^{-2}$ . Compaction of the DNA bound to the nanorods is achieved by incubation of the nanorods in a solution of  $\text{CaCl}_2$ .

*In vitro* transfection experiments are carried out with the Human Embryonic Kidney (HEK293) mammalian cell line using the GFP and luciferase reporter genes, respectively. For transfection, the nanorods are incubated with HEK293 cells for 4 hours in Opti-MEM cell culture medium. Transmission electron microscope images and EDX analysis showed that the nanorods are located in the vesicles or the cytoplasm but not the nucleus. This indicates that the transfection is due to plasmids released or cleaved from the nanorods before nuclear entry. Nanorods with compacted plasmids displayed a 255-fold increase in luciferase expression in comparison to naked DNA. GFP-positive cells by nanorod transfection were 4 times higher than that achieved by naked DNA. Compared to nanorods with compacted plasmids alone, bi-functional nanorods conjugated with transferrin increased luciferase expression by a factor of 3.4 and GFP expression from 11% to 22%. Further evidence that transferrin was promoting receptor-mediated endocytosis of the nanorods is seen by an increase in GFP expression to 27% and increased luciferase expression by a factor of 1.9 in the presence of chloroquine in the cell culture medium. To confirm that transfection was due to intracellular rather than extracellular

release of plasmids, nanorods complexed with the luciferase-plasmid were incubated in serum-containing media. The supernatant was removed at varying time points from 15 minutes to 4 hrs and used to transfect the HEK293 cells. In all cases no significant transfection above background could be detected in these samples. This data suggests that the transfection detected is a result of the intracellular released plasmids from the 200 nm nanorods. Dual component rods 20  $\mu\text{m}$  long by 170 nm width could not transfect the HEK293 cells as efficiently as the 200 nm nanorods under otherwise identical conditions suggesting that size of these nanorods is a critical parameter in influencing transfection efficiency. Furthermore, transferrin conjugation and chloroquine addition does not improve transfection with the 20 micron nanorods indicating that the increase in size may be preventing or altering the route of uptake of the nanorods. The dependence of transfection efficiency on particle size has been recently reported and is clearly an area of research that warrants further investigation.[6] Thus, the capacity to tailor dual component nanorods to defined uniform sub-micron sizes may afford this delivery system an important advantage over many alternative non-viral gene delivery systems.

Current and future work is focused on optimizing the nanorod gene delivery system. In addition, the unique properties of the nanorods that allow for defined aspect ratios and the addition of magnetic segments are being utilized as a probe to determine the effects and inter-relationships of size, magnetism and cell targeting proteins in gene delivery. Finally, in collaboration with the Department of Pathology at The Johns Hopkins School of Medicine, nanorods are currently being evaluated for their potential in genetic immunization using Ovalbumin as a model protein.

## METHODS AND MATERIALS

The nanorods are fabricated by electrodeposition into an  $\text{Al}_2\text{O}_3$  template (Anodisc, Whatman) with a pore diameter of 100 nm.[7] An evaporated silver film on one side of the template serves as the working electrode in a three-electrode configuration. A thin layer of silver is electrodeposited into the template from 50 mM  $\text{KAg}(\text{CN})_2$ , 0.25 M  $\text{Na}_2\text{CO}_3$  buffered to pH 13 at a potential of  $-1.0\text{V}$  (Ag/AgCl) to ensure easy release of the nanorods from the template. The Au segments are deposited from a commercial gold plating solution (Technic Inc.) at a potential of  $-1.0\text{V}$  (Ag/AgCl) and the Ni segments are deposited from a solution of 20  $\text{g l}^{-1}$   $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ , 515  $\text{g l}^{-1}$   $\text{Ni}(\text{H}_2\text{NSO}_3)_2 \cdot 4\text{H}_2\text{O}$ , 20  $\text{g l}^{-1}$   $\text{H}_3\text{BO}_3$  buffered to pH 3.4 at a potential of  $-1.0\text{V}$  (Ag/AgCl). The gold segments are deposited before the nickel segments in order to ensure that the nickel segments were not etched by the nitric acid during removal of the silver. The silver layers are dissolved in 70vol % nitric acid and the alumina template is then dissolved in 2 M potassium hydroxide. The nanorods are washed repeatedly using 2 M potassium hydroxide, de-ionized water and ethanol.

150  $\mu\text{l}$  of 0.1 M AEDP (Pierce) solution is added to 200  $\mu\text{l}$  aliquots of nanorods ( $\sim 1 \times 10^6$ ) suspended in distilled water. Following incubation for 24 h and washing, 2  $\mu\text{g}$  of plasmid is added to each aliquot of nanorods, (pH 5.7) and incubated at 4  $^\circ\text{C}$  for 24 h. After washing, 2  $\mu\text{l}$  of a 2M  $\text{CaCl}_2$  solution is added to each aliquot and then incubated for 24 h at 4  $^\circ\text{C}$ . For fluorescent staining of plasmids, nanorods are incubated with 100  $\mu\text{l}$  of 0.01  $\text{mg/ml}^{-1}$  Hoechst 33258 followed by washing. 5 mg of rhodamine-conjugated transferrin (Molecular probes) in

PBS with 5 mM EDTA is reacted with 120  $\mu\text{l}$  of 5 mg  $\text{ml}^{-1}$  iminothiolane (Pierce) for 30 minutes at room temperature. The protein is purified by dialysis at 4 °C. Twenty  $\mu\text{l}$  of 5 mg  $\text{ml}^{-1}$  rhodamine-transferrin-SH is added to each aliquot of nanorods and incubated for 24 h at 4°C.

HEK293 cells (ATCC) are cultured in T75 flasks in DMEM with 10% FCS and ABAM. All cell culture and Lipofectamine reagents are purchased from Gibco BRL, Rockville, MD. The serum-containing media is replaced every 3 days and split 1:3 at pre-confluence. HEK293 cells are seeded onto 24-well plates ( $3 \times 10^5$  cells/well) for transfection using the luciferase plasmid (Vical), 12-well plates ( $8 \times 10^5$  cells/well) for transfection using the GFP-plasmid (Clontech Incorporated) and 6-well plates ( $2 \times 10^6$  cells/well) for TEM studies. Each well (24-well) is transfected in 0.5 mL reduced-serum Opti-MEM media. Selected wells are incubated with Opti-MEM containing 100  $\mu\text{M}$  chloroquine. Lipofectamine: DNA complexes at a ratio of 4:1 using 8  $\mu\text{g}$  Lipofectamine in 40  $\mu\text{L}$  Opti-MEM and 2  $\mu\text{g}$  DNA in 40  $\mu\text{L}$  Opti-MEM is added to control wells. 40  $\mu\text{L}$  of the nanorods/DNA suspension is added per well. After 4 hours, the transfection media is removed and the cells washed. After 2 days of further incubation in serum-containing media, wells are washed with phosphate buffered saline (PBS) and imaged live. Transfection with the luciferase plasmid followed the same protocol for GFP plasmid with relative light units (RLU) measured using a luminometer (EG&G Berthold MiniLumat) and normalized to protein content using the BCA protein assay (Biorad).

For TEM studies on internalization, cells are fixed with 2% glutaraldehyde/ 2% paraformaldehyde in PBS at selected time-points. After washing with PBS, cells are dehydrated with graded ethanol and coated with 2% osmium tetroxide (Aldrich). TEM samples are sectioned in epoxy resin by microtome and developed using 2% uranyl acetate and 0.04% lead citrate. TEM-EDX analysis is performed using a spot size of 5nm with a Philips CM-300 FEG with EDX attachment.

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